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DNA Signal Architecture for Transcriptional Activation by the Regulatory Protein VirG

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Transcription of the virulence genes of *Agrobacterium* Ri and Ti plasmids is activated by the regulatory protein VirG in response to plant factors. The minimal DNA unit recognized by the VirG protein is six-base-pair sequences called "vir box". In the upstream regions of the virulence genes, one to four vir boxes are repeated in the same direction, and the most upstream vir box is always preceded by an additional vir box in an inverted orientation. Organization of vir boxes, however, differs with the virulence genes. To clarify signal architectures essential for the transcriptional activation, synthetic promoters with systematically arranged vir boxes were examined in *Agrobacterium* cells on transcription induced by a plant factor. The helically phased vir boxes preceded by the inverted vir box promoted inducible transcription, and this helical phase relative to that of the -35 and -10 regions of the promoter was critical. In addition, four directly repeated vir boxes without the preceding inverted one induced transcription at a significant level. These results clearly indicate that the appropriately phased vir boxes are necessary and sufficient for transcriptional activation. This finding is consistent with the view that interaction of the VirG protein with RNA polymerase required for transcriptional activation occurs in the -35 region of the promoter though the upstream inverted vir box appears to enhance and stabilize interaction between the VirG protein and DNA.

KEY WORDS: *Agrobacterium* / *cis* element / DNA-protein interaction / promoter / vir box

INTRODUCTION

The soil bacteria *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* confer hairy roots and crown gall, respectively, on most dicotyledonous plants. These phenomena are caused by the transfer of a DNA segment (T-DNA) of bacterial Ri or Ti plasmid to the plant nuclear genome followed by synthesis of plant phytohormones directed by the integrated T-DNA. The T-DNA transfer is promoted by the *trans*-acting functions encoded by the virulence genes (*vir*) (for review see refs. 1 and 2). Expression of most of the *vir* genes is positively regulated at the transcriptional stage.[3-7] This activation is triggered by plant phenolic compounds such as acetosyringone, being mediated through the receptor protein VirA and the transcriptional activator protein VirG.[8-17] DNA sequences upstream from inducible *vir* transcripts on Ri and Ti plasmids have structural features as follows. The consensus structure of bacterial promoters is less conserved in the inducible *vir* promoters, particularly at their -35 regions. Each upstream region contains one to four copies of characteristic six-base-pair (bp) blocks with the consensus sequence of 5' TG(A/T)AA(T/C)3' (vir box). These vir boxes are phased at an interval of integral multiples of 11 bp. Moreover, their helical

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phase is nearly opposite to that of the -35 and -10 regions of the inducible promoters, and the most upstream vir box is invariably preceded by an additional vir box in the inverted orientation.[7,12] Footprinting experiments *in vitro* by DNase I protection and dimethyl sulfate methylation inhibition with the purified VirG protein indicate that the VirG protein is bound to the phased vir box sequences from the major groove along one side of double-helix DNA.[13-15]

Although the vir box is conspicuous to be a unit recognized by the VirG protein *in vitro*, the vir box arrangement prerequisite to the transcriptional activation is still equivocal because overall organization of the vir boxes varies with the *vir* genes. A set of an inverted vir vox and a regular vir box is followed by directly repeated vir boxes for most *vir* genes but not for *virD* and *virE*. Moreover, in the cases of *virC* and *virG* promoters, the -35 region is flanked by two directly repeated vir boxes. Initial binding of the VirG protein occurs at or near the inverted repeats[13] present in an upstream region of every inducible promoter,[4,7] implying that the inverted repeats are essential structure for transcriptional activation. However, it has been speculated that the actual site for leading RNA polymerase by the VirG protein is around the -35 region to which cooperative binding of VirG molecules extends.[12,13] In order to verify their potentiality, we have now prepared synthetic promoters with systematically arranged vir box sequences and examined their ability to induce transcriptional activation in *Agrobacterium* cells.

MATERIALS AND METHODS

General procedures. Standard procedures for recombinant DNA experiments (plasmid isolation, DNA cleavage with restriction enzymes, ligation, DNA sequencing, *etc.*) were as described.[16-18] Oligodeoxyribonucleotides were synthesized using a Beckman DNA Synthesizer.

Induction by a plant factor. The *Agrobacterium* strain AR1007[17] carrying Ri plasmid A4 (pRiA4) was grown to 3×10^8 cells/ml in YEB medium,[16] collected by centrifugation, and then suspended in one-half the original volume of MSMES medium, which was MS medium supplemented with 20 mM MES-Good's buffer (pH 5.5). To one portion of the cell suspension, acetosyringone was added to 0.2 mM (inducing condition); the other portion was used as a control (non-inducing condition). Cells under both conditions were cultured with aeration at 28℃ for 24 hr.

β - Galactosidase assay. β - Galactosidase activity was measured basically as described by Miller.[19] Bacterial cells cultivated as above were collected and suspended in 1 ml of Z-buffer and its bacterial density was determined from the absorbance at 600 nm (A_{600}). The cell suspension was vigorously mixed with one drop of toluene. The reaction was started by adding 0.2 ml of *o*-nitrophenyl- β - D-galactopyranoside (4 mg/ml), and the mixture was incubated at 28℃ for 40 min. After stopping the reaction by addition of 0.5 ml of a 1 M Na_2CO_3 solution, the absorbance at both 420 nm (A_{420}) and 550 nm (A_{550}) for each reaction was measured. Specific activities of β-galactosidase were calculated using the following formula:

$$\text{Units} = 1000 \times (A_{420} - 1.75 \times A_{550}) \,/\, (40 \text{ min} \times A_{600})$$

RESULTS

Construction of the promoters carrying various vir box arrangements

A shuttle vector, pVBR-0, was the starting plasmid from which all plasmids analyzed in this study were derived. This plasmid primarily consisted of the *Escherichia coli lacZ* gene as a reporter, the kanamycin resistant mini-Ti plasmid,[20] and the ColE1 replication origin

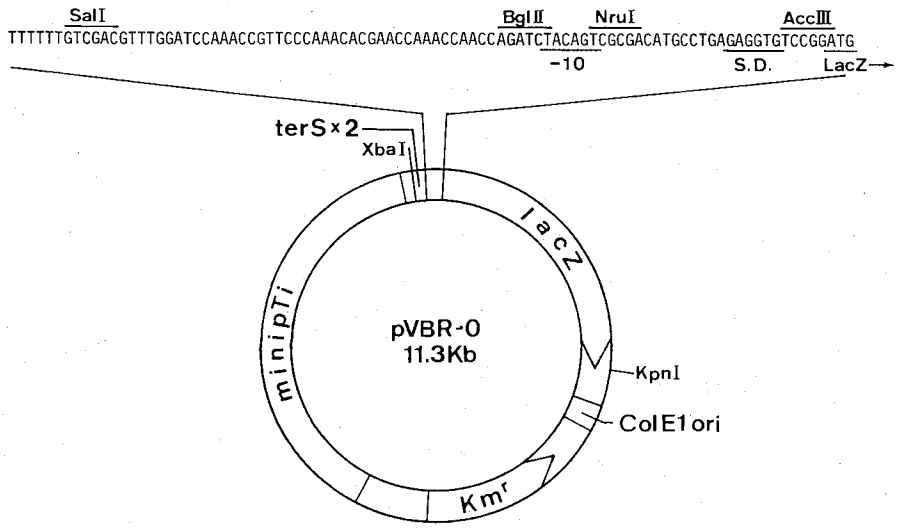

Figure 1. Structure of the starting plasmid carrying the synthetic promoter sequence. The location of the β-galactosidase gene (lacZ), the replication origin of the ColE1 plasmid (ColE1 ori), kanamycin resistance gene (Km^r), the mini-Ti plasmid region (mini-pTi), and the transcription terminator of phage fd (terS X 2) are indicated on the circular map of the starting plasmid pVBR-0. The sequence of the synthetic promoter region is shown above the circular map. The -10 region of the promoter (-10), the Shine-Dalgarno sequence (S.D.), and the start codon for *lacZ* translation are indicated by underlines. The unique restriction sites are indicated on the sequence or the circular map.

region[16] (see Figure 1). Nucleotide sequence of the promoter region upstream from the reporter gene was designed to contain neither irrelevant potential -10 region sequences for eliminating impertinent transcription initiations nor latent -35 region sequences for decreasing basal level transcription exerted by the promoter. Besides, the transcription terminator sequence of phage fd (*terS*)[21] was tandem put at a site upstream from the promoter to exclude possible read-through transcripts. Synthetic DNA fragments containing various vir box arrangements were substituted for the *Sal* I-*Bgl* II segment of pVBR-0. Nucleotide sequence of those fragments are shown in Figure 2, together with the promoter sequence. The promoters carried by pVBR-IT and pVBR-2T contained a set of inverted repeat vir boxes and a set

Figure 2. DNA sequences of the synthetic promoters. The DNA sequences of the promoters constructed in this study are shown. The -35 and -10 regions are marked by double lines below the sequences. Regular and inverted vir box sequences are indicated by thick underlines.

of regular repeat vir boxes flanking the -35 region, respectively. The promoters in pVBR-I3T, pVBR-I3TL, and pVBR-I3TS had the same combined architecture in which one inverted vir box preceded three phased vir boxes, but the spacer between the vir box array and the -10 region was 1-bp longer in pVBR-I3TL or 1-bp shorter in pVBR-13TS than that in pVBR-I3T. In pVBR-4T, four vir boxes were helically phased but lacking an inverted vir box.

Transcriptional activation exerted by the promoters including various vir box arrangements

The plasmids constructed above were separately introduced into the *Agrobacterium* strain AR1007. The resulting transformants were cultivated under the inducing conditions and the non-inducing conditions, and the level of β- galactosidase directed by each test promoter was measured (see Materials and methods). The results are summarized in Figure 3. Although inducible expression of β- galactosidase did not occur with the promoter carrying either the inverted vir boxes alone (pVBR-IT) or the tandem vir boxes alone (pVBR-2T), but did occur at a significant level with the promoter carrying both of them (pVBR- I3T). The promoter including four phased vir boxes but lacking an inverted vir box (pVBR-4T) showed the ability to induce expression at a comparable level. pVBR-I3TL carrying 1-bp insertion in the spacer was competent for inducible expression at a level comparable to that of the parental pVBR-I3T, while pVBR-I3TS carrying 1-bp deletion in the spacer was completely deficient in transcriptional activation.

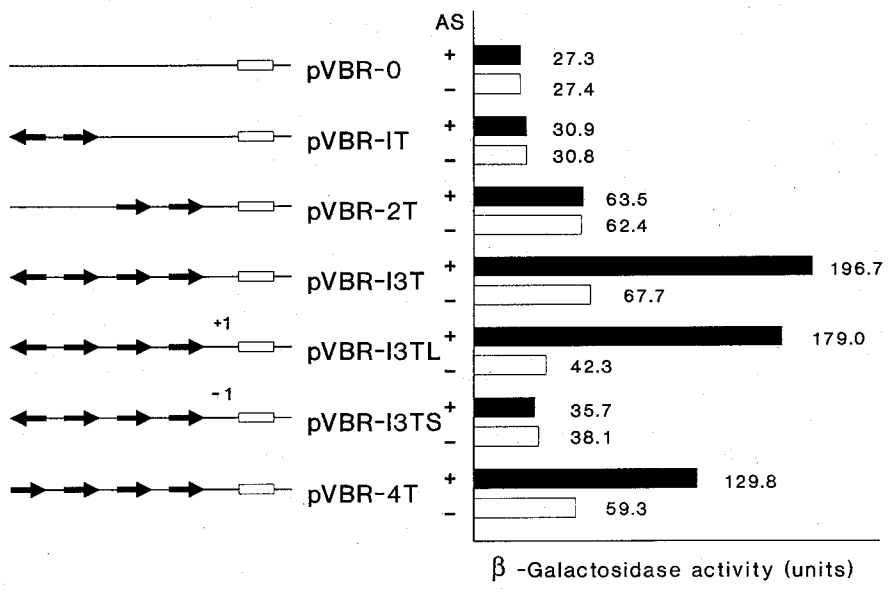

Figure 3. β - Galactosidase expression directed by the synthetic promoters. The arrangement of vir boxes (arrows) and the -10 regions of the promoters (open boxes) are illustrated on the left side of the plasmid name. +1 and -1 show 1-bp insertion and 1-bp deletion, respectively, in the spacer region between the vir box array and the -10 region. The β - galactosidase activities with bacterial cells grown under the inducing and non-inducing conditions (+ AS and − AS; AS = acetosyringone), are shown by filled and open bars, respectively, with numerals in unit on the right side of the plasmid name.

DISCUSSION

In this study, we constructed the artificial promoters with various vir box arrangements and examined their ability to induce transcriptional activation upon acetosyringone in *Agrobacterium* cells carrying pRiA4. Since there are several lines of circumstantial evidence indicating that transcription directed by the inducible *vir* promoter can be monitored accurately at the translation level (e.g. refs. 5, 7, 8), the activity of the reporter gene product, β-galactosidase, measured in this study should reflect the transcription level directed by the test promoters. Therefore, the results shown in Figure 3 clearly indicate that neither the upstream inverted repeat vir boxes alone, nor the two regular repeat vir boxes flanking the -35 region alone (pVBR-IT and pVBR-2T) were able to function in response to acetosyringone, but the promoter carrying the combined architecture (pVBR-I3T) led to transcriptional activation. These results are consistent with our previous interpretation that all vir boxes including the inverted one are recognized by the VirG protein, being substantial

as the *cis*-acting elements for transcriptional activation. However, there are natural inducible *vir* promoters that are accompanied only by one set of the inverted repeat vir boxes as pVBR-IT.[4,7] This inconsistency may imply that the intrinsic activity of the used synthetic promoters is too weak to direct enough transcriptional activation because the highest level of β-galactosidase observed in this study (about 200 units) is considerably lower than that exerted by a natural *vir* promoter (over 500 units);[22,23] otherwise nucleotide sequences of short spans outside vir boxes (e.g. -35 region sequence), though the individuals are dispensable, might moderately and additively affect transcriptional activation.

Effects of the relative phase between the phased vir boxes and the -10 region sequence were examined with three similar plasmids, pVBR-I3T, pVBR-I3TL, and pVBR-I3TS. The former two inducibly synthesized β-galactosidase at comparable levels, while the latter one showed no ability to induce expression. These results coincide well with the phase variation seen in the native *vir* promoters.[7] The inducible *vir* promoters of pRiA4 have either pVBR-I3T type structure (the *virB*, *virC*, and *virG* promoters) or pVBR-I3TL type structure (the *virA* and *virE* promoters), and no inducible *vir* promoter belonging to the class of pVBR-I3TS has been found so far.[5,7] Therefore, the relative phase between the vir box array and the -10 region is flexible only within fairly restricted limits, thereby allowing that the VirG protein and RNA polymerase simultaneously approach DNA from either side.[12,13]

The most interesting result in this report was that the promoter accompanying the four phased vir boxes but lacking an inverted vir box was active for inducible expression, though the induction level was slightly lower than that of pVBR-I3T. Since no potential sequences for an inverted vir box are present in the upstream region of the phased vir boxes, it can be said that the inverted repeat is not essential for inducible expression; or else multiple direct repeats of the vir box functionally overcome the lack of an inverted vir box. This fact supports our previous hypothesis that although the inverted repeat is required for enhancement and stabilization of interaction between the VirG protein and DNA, the actual guiding of RNA polymerase by the VirG protein occurs at a more downstream region (presumably in the -35 region) after cooperative binding of the VirG protein has enough spread over this region. An *in vitro* transcription system, consisting of three proteins (RNA polymerase, the VirG protein, and the VirA protein), that we have recently developed,[24] will be useful to completely prove this hypothesis.

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